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Cell cycle inhibitory activity of *Piper longum* against A549 cell line and its protective effect against metal-induced toxicity in rats

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Anticancer potential of *Piper longum* fruit against human cancer cell lines (DU-145 prostate, A549 lung, THP-1 leukemia, IGR-OVI-1 ovary and MCF-7 breast) as well as its *in vitro* and *in vivo* biochemical efficacy in $AlCl_3$-induced hepatotoxicity were evaluated in the rats. Dried samples were extracted with several solvents using soxhlet apparatus. Flavonoid content in chloroform, benzene, ethyl alcohol and aqueous extracts of fruit was 19, 14, 12 and 11 µg quercetin equivalent/mg of sample, respectively. Hexane extracts exhibited 90-92% cytotoxicity against most of the test cell lines (A549, THP-1, IGR-OVI-1 and MCF-7), while benzene extract displayed 84-87% cytotoxicity against MCF-7, IGR-OV-1 and THP-1 cell lines. Among extracts, hexane, benzene and acetone extracts demonstrated considerable cytotoxicity (91-95%) against A549 (lung cancer) cell line in Sulforhodamine B dye (SRB) assay. Cell cycle analysis revealed that hexane, benzene and acetone extracts produced 41, 63 and 43% sub-G1 DNA fraction, demonstrating cell cycle inhibitory potential of these extracts against A549 cell line. Chloroform, ethyl alcohol and aqueous extracts displayed 71, 64 and 65% membrane protective activity, respectively in lipid peroxidation inhibition assay. *P. longum* fruit extracts also ameliorated $AlCl_3$-induced hepatotoxicity, as indicated by alterations observed in serum enzymes ALP, SGOT and SGPT activity, as well as creatinine and bilirubin contents. In conclusion, study established the cytotoxic and hepatoprotective activity in *P. longum* extracts.

Keywords: Cytotoxicity, Cell cycle, *Piper longum*, Lipid peroxidation, Hepatoprotective, Hepatotoxicity, Serum enzymes.

Piper longum (Piperaceae) has been used in the Indian traditional medicine system for treatment of various diseases, including cancer. It is a slender aromatic climber with perennial woody roots having wide geographical distribution in India from central Himalaya to Assam, lower part of Bengal and evergreen forest of western Ghats. Various medical attributes have been documented for the fruits of *P. longum* against ailment of respiratory tract viz., cough, bronchitis, asthma, epilepsy and insomnia[1,2].

Cancer has remained as second cause of death next to cardiovascular disease in humans. About 7.6 million people died due to cancer in the world during 2007[3]. Hence, much attention is being paid to explore novel anticancer drugs[4]. The development of cancer is associated with disorders in the regulation of the cell cycle and cellular DNA damage. Cells have developed several defense mechanisms to cope with the constant attack on their DNA, for example direct repair, halting cell-cycle progression or apoptosis[5].

Plants have been regarded as a potential source of cancer chemoprevention drug discovery and development. Plant-based diet can result in reduction of cancer markers without using conventional treatment[6]. Phytochemicals, such as flavonoids, terpenes, alkaloids etc. have received considerable attention in recent years due to their diverse pharmacological properties, including cytotoxic and cancer chemopreventive effects[7]. *Punica granatum* fruit extract has shown cell cycle arrest potential against human pancreatic cancer cells[8]. Similarly, some plant extracts also arrest cell cycle in different cancer cell lines[9,10].

Imbalance between antioxidant defense and reactive oxygen species (ROS) causes oxidative stress, a pathophysiological condition, leading to

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Abbreviations: ALP, alkaline phosphatase; BHA, butylated hydroxyanisole; FACS, fluorescence activated cell sorting; LPOI, lipid peroxidation inhibition; MDA, malondialdehyde; PBS, phosphate buffered saline; QE, quercetin equivalent; ROS, reactive oxygen species; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamate pyruvate transaminase; SRB, sulforhodamine B dye; TCA, trichloro acetic acid.

many human diseases, including cancer, aging and atherosclerosis[2]. Free radicals cause peroxidation of unsaturated membrane lipids and disrupt their integrity, resulting into impaired functioning of cell. Lipid peroxidation is thus attracting much attention, due to its involvement in physiological and pathological conditions of liver, kidney and brain toxicity. Plant-derived antioxidants have capability to scavenge free radical and thereby inhibit the lipid peroxidation[7,11].

Liver is a largest gland and versatile organ of body that regulates internal chemical environment. Hepatocytes play a central role in the metabolism of alcohol or drugs which may enhance the ROS production. Excessive metabolism or metabolism of toxic metabolite producing substrates results into the overproduction of ROS and oxidative stress in the hepatocytes[12]. The reason hepatocytes have the highest antioxidant function, as compared with the cells of other organs is probably that oxidative stress is easily induced in the hepatocytes. Aluminum chloride ($AlCl_3$) catabolized radical-induced lipid peroxidation damages the membranes of liver cells and organelles, causes the swelling and necrosis of hepatocytes and results in the release of cytosolic enzymes[13]. $AlCl_3$-induced liver injury has been recognized as a potentially toxic problem. Al is widely distributed in the environment and is extensively used in daily life, which causes its easy exposure to human beings[13].

Herbal medicines have been used in the treatment of liver diseases for a long time. A number of herbal preparations are available in the market. Some commonly used hepatoprotective herbal preparations are *Phyllanthus*, *Silybum marianum* (milk thistle), glycyrrhizin (licorice root extract) and Liv52 (mixture of herbs)[14,15]. In the present communication, we have investigated the anticancer potential of various extracts of *Piper longum* fruit against many cancer cell lines and have evaluated cell cycle inhibitory activity against A549 (lung cancer) cell line. The *in vitro* as well as *in vivo* biochemical efficacy of *P. longum* in $AlCl_3$-induced hepatotoxicity has also been investigated.

Materials and Methods

Plant material and preparation of extracts

Piper longum fruits were purchased from local market and authenticated by experts in the Botany Department, University of Allahabad, Allahabad. The samples were surface-sterilized, crushed and ground into fine powder. Powdered material was sequentially extracted with hexane, benzene, chloroform, ethyl acetate, acetone, ethyl alcohol and water in Soxhlet apparatus as described earlier[16,17]. The respective extract fractions were centrifuged (2000 *g*, 5 min), filtered and lyophilized. The dried residues were dissolved in DMSO or in respective solvents to assess their biological activities.

Cell lines, growth conditions and treatment

Human cancer cell lines, namely prostate (DU-145), lung (A549), leukemia (THP-1), ovary (IGR-OVI-1) and breast (MCF-7) were procured from National Center for Cell Sciences, Pune, India. Cell lines were grown and maintained in RPMI-1640 medium, pH 7.4 with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Cells were grown in CO_2 incubator (Heraeus, GmbH Germany) at 37°C in the presence of 90% humidity and 5% CO_2.

Cytotoxic assay by Sulforhodamine B dye (SRB Assay)

The *in vitro* cytotoxicity of the extracts was determined using sulforhodamine-B dye (SRB) assay[18]. Cell suspension (100 µl, 1×10^5 to 2×10^5 cells per ml, depending upon mass doubling time of cells) was grown in 96-well tissue culture plate and incubated for 24 h. Stock solutions of test extracts were prepared in DMSO and serially diluted with growth medium to obtain desired concentrations. The test extract (100 µg/well) was then added to the wells and cells were further incubated for another 48 h. The cell growth was arrested by layering 50 µl of 50% trichloro acetic acid (TCA) and incubated at 4°C for 1 h, followed by washing with distilled water and then air-dried. SRB (100 µl, 0.4% in 1% acetic acid) was added to each well and plates were incubated at room temperature for 30 min. The unbound SRB dye was washed with 1% acetic acid and then plates were air-dried. Tris-HCl buffer (100 µl, 0.01 M, pH 10.4) was added and the absorbance was recorded on ELISA reader at 540 nm. Each test was done in triplicate. The values were reported as mean ± SD of three replicates.

Cell cycle analysis

Nuclear DNA at sub-G1 phase in normal and extract-treated A549 cancer cell lines was estimated by cell cycle analysis using Flow-cytometer. A549 cell lines (5×10^5/ml) were treated with sequential extracts of *P. longum* (100 µg/ml) for 24 h and washed twice with ice-cold phosphate buffered

saline (PBS), harvested, fixed in cold 70% ethanol in PBS and stored at - 20°C for 30 min. After fixation, the cells were incubated with RNase A (0.1 mg/ml) at 37°C for 30 min and then stained with propidium iodide (50 µg/ml) for 30 min on ice in dark[19]. Nuclear DNA content was measured by using BD-LSR Flow cytometer (Becton Dickinson, USA) equipped with electronic doublet discrimination capability using blue (488 nm) excitation from argon laser. The fluorescence intensity of sub-G1 cell fraction represented the apoptotic cell population. Camptothecin (5 µM) was used as positive control.

Determination of flavonoid content

$AlCl_3$ colorimetric method[20] as modified by us[21] was used for determination of flavonoid content in test extract. Small amount (0.2 ml) of extract in DMSO (2 mg/ml) was taken, followed by addition of methanol (1.8 ml), 10% $AlCl_3$ (0.1 ml), 1 M potassium acetate (0.1 ml) and distilled water (2.8 ml). Contents were mixed, incubated at room temperature for 30 min and then absorbance was measured at 415 nm. Calibration curve was prepared with quercetin and the amount of flavonoids in the test sample was expressed as µg quercetin equivalent/mg sample (µg QE/mg). Experiments were performed in triplicate and the results were expressed as mean ± SEM.

Lipid peroxidation inhibition (LPOI) assay

The lipo-protective efficacy of extract was estimated by the method of Halliwell and Gutteridge[11] with some modification[22]. The liver tissues were isolated from normal albino Wistar rats and 10% (w/v) homogenate was prepared in phosphate buffer (0.1 M, pH 7.4 having 0.15 M KCl) using homogenizer at 4°C. The homogenate was centrifuged at 800 *g* for 15 min and clear cell-free supernatant was used for *in vitro* lipid peroxidation inhibition assay. 100 µl of extract (2 mg/ml) dissolved in respective solvents was evaporated to dryness, followed by addition of 1 ml KCl (0.15 M) and 0.5 ml of tissue homogenate. Peroxidation was initiated by adding 100 µl $FeCl_3$ (0.2 mM). After incubation at 37°C for 30 min, lipid peroxidation was monitored by the formation of thiobarbituric acid reactive substances which were estimated by adding 2 ml of ice-cold hydrochloric acid (0.25 N) containing 15% TCA, 0.38% thiobarbituric acid (TBA) and 0.5% butylated hydroxytoluene (BHT). The reaction mixture was incubated at 80°C for 1 h, followed by cooling and centrifugation (1000 *g*, 5 min). The absorbance of pink supernatant was measured at 532 nm. Butylated hydroxyanisole (BHA) was used as standard for comparison. All analyses were carried out in triplicate and results were expressed as mean ± SD. The protective effect of extracts against lipid peroxidation (% LPOI) was calculated by using the following formula:

$$\% \text{ LPOI} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of control and A_1 is absorbance in the presence of the sample/standard compounds. The results were expressed as mean ± SD of three replicates.

Assessment of hepatic markers in aluminum chloride induced rats

Animal model and in vivo experimental protocols

Wistar rats weighing 150-180 g of either sex were procured from CDRI, Lucknow and kept in departmental animal house in well cross (23 ± 2°C) with light and dark cycles of 12 h of 1 week before and during experiments. Animals were provided with standard rodent pellet diet (Amrut, India) and water was given *ad libitum*. All *in vivo* studies were performed in accordance with the guide for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Care Committee, CPCSEA, India.

The rats were divided into four groups (I-IV) with six in each group. Group I received saline and served as healthy control. Hepatotoxicity was induced by the oral administration of $AlCl_3$ (100 mg/kg body wt in saline) for 21 days in Groups II-IV. Liv-52 (Himalayan Drug Company, India), the known hepato-protective drug was administered daily in group III at a dose of 50 mg/kg body wt. *P. longum* fruit extract-treated group (Group IV) received aqueous extracts at dose of 200 mg/kg body wt. After experimental period (21 days), all animals were sacrificed and the blood was collected for evaluation of biochemical parameters.

Collection of blood and isolation of serum

Heart was punctured and 5 ml of blood was drawn. The blood was allowed to clot and serum was separated at 2500 rpm for 15 min. Serum was transferred into separate eppendorf tubes and stored at -70ºC, until analysis.

Effect of extracts on serum hepatic markers

The biochemical parameters viz., alkaline phosphatase (ALP, U/L), serum glutamic oxaloacetic

transaminase (SGOT, U/L), serum glutamate pyruvate transaminase (SGPT, U/L), bilirubin (total and direct) and creatinine were assayed by using commercially available kits (Erba Diagnostic Kits, Germany).

Statistical analysis

All experiments were carried out in triplicate. Results were expressed as mean ± standard error of mean (SEM) and mean ± standard deviation (SD). The plots were prepared using GraphPad Prism software. One-way and two-way ANOVA were used for statistical analysis. P values of less than 0.05 were considered significant.

Results

Cytotoxic activity of *P. longum* fruit extracts

The cytotoxicity activity of *P. longum* fruit extracts was tested against five cancer cell lines, namely DU-145 (prostate), A549 (lung), IGR-OV-1 (ovary), MCF-7 (breast) and THP-1 (leukemia) at the concentration of 100 µg/mL using SRB assay and results are shown in Fig. 1A and B. Significant inhibitory potential was observed in most of the test extracts. Hexane, benzene and acetone extracts demonstrated appreciable cytotoxicity (91-95%) against A549 cell line. Similarly, hexane and benzene extracts exhibited 84-92% growth inhibition of MCF-7 and IGR-OV-1 cell lines. Most of the extracts produced noticeable growth inhibition (76-90%) of THP-1 cell lines. Hexane fraction of *P. longum* accounted for 80% cytotoxicity against DU-145 cell line. Ethyl acetate and acetone extracts showed 65-74% growth inhibition potential against breast cancer cell line. Rest of the fruit extracts showed low to moderate (11-60%) cell growth inhibition of test cell lines at test concentration. Standard anticancer drugs exhibited 51-67% cytotoxicity against different test cell lines.

Cell cycle analysis

Deregulated cell cycle progression in cancer cells is an effective strategy to inhibit tumor growth. Therefore, effect of potent cytotoxic fractions of *P. longum* fruit on cell cycle progression in A549 cells was examined. Cells were treated with test extract at the concentration of 100 µg/mL for 24 h and fluorescence activated cell sorting (FACS) analysis was done. The DNA histogram showed that extract treatment increased hypodiploid sub-G1 DNA fraction (<2nDNA) (Fig. 2A-C). Treatment with hexane, benzene and acetone extracts at 100 µg/mL increased the sub-G1 population up to 41, 63 and 43%, respectively. Standard anticancer drug camptothecin at 5 µM concentration produced 48% increase in sub-G1 DNA fraction (Fig. 2 D), while in untreated cells, the sub-G1 DNA fraction was 3.5% only (Fig. 2E).

Quantitative determination of flavonoid content

Maximum flavonoid content was found in chloroform extract (19.67 ± 1.45 µg QE/mg), followed by benzene (14.33 ± 0.67 µg QE/mg). Hexane extract was devoid of flavonoid content. The

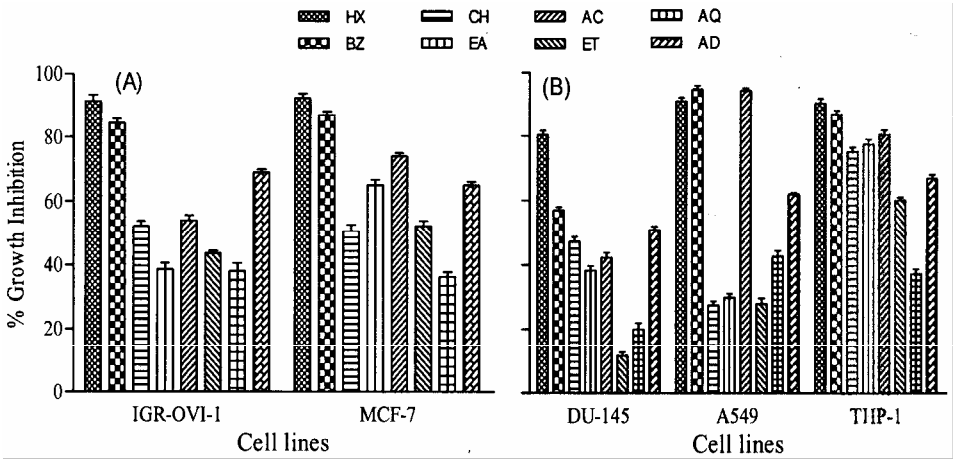

Fig. 1—Cytotoxic activity of *P. longum* fruit extracts against (A) ovary and breast, (B) prostate, lung and leukemia cell lines using SRB assay [Extracts were prepared in hexane (HX), benzene (BZ), chloroform (CH), ethyl acetate (EA), acetone (AC), ethyl alcohol (ET) and water (AQ) as described in 'Materials and Methods'. Cytotoxic potential of extracts was measured against DU-145 (prostate), A549 (lung), IGR-OVI-1 (ovary), MCF-7 (breast) and THP-1 (leukemia) human cancer cell lines in presence of 100 µg of extract per well. Results are expressed as mean ± SD of three replicates ($p<0.05$). *Abbreviation*: AD, anti cancer drugs [mitomycin C (10 µM) against DU-145, paclitaxel (10 µM) against MCF-7, adriamycin (1 µM) against IGR-OVI-1, 5-fluorouracil (20 µM) against THP-1 and A549]

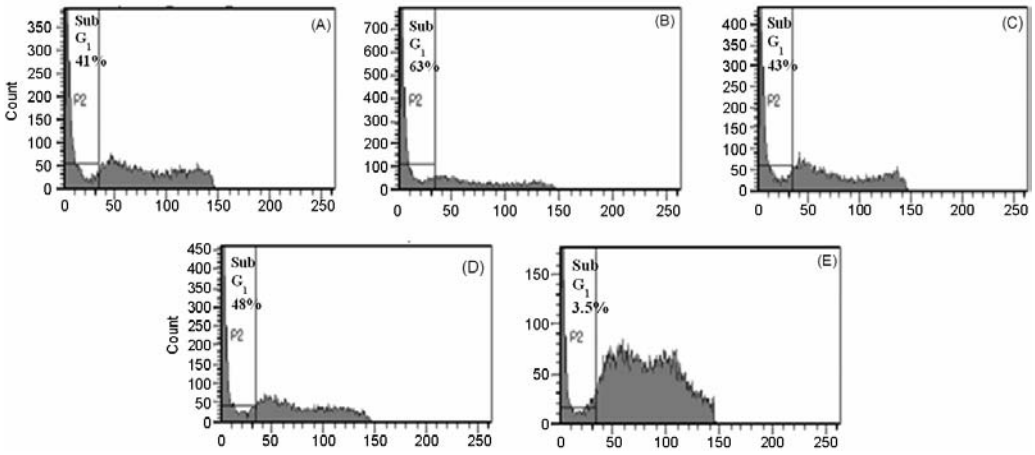

Fig. 2—Cell cycle analysis of A549 cells treated with *P. longum* fruit (A) hexane extract, (B) benzene extract, (C) acetone extract, (D) camptothecin (5 µm) and (E) untreated cells [A549 Cells (5 x 10^5 cells/ml/well) were exposed to extracts (100 µg/well) and camptothecin for 24 h and then stained with propidium iodide to determine DNA fluorescence and cell cycle phase distribution as described in 'Materials and Methods'. Fraction of cells for sub-G1 population was analyzed from FL2 *vs.* cell counts. Data are representative of one of three similar experiments]

order of flavonoid contents in extracts was chloroform, benzene, ethyl alcohol (12.00 ± 5.8), water (11.67 ± 0.88), acetone (8.33 ± 0.33), ethyl acetate (2.53 ± 0.15 µg QE/mg) and hexane.

Lipid peroxidation inhibition activity

The liver homogenate of albino Wistar rats undergo rapid peroxidation when incubated separately with $FeCl_3$. The iron-induced production of peroxide in turn attacks the biological material, leading to the formation of malonodialdehyde (MDA) and other aldehydes which form a pink chromogen with TBA showing maximum absorbance at 532 nm[11]. Significant *in vitro* anti-lipid peroxidative activity was displayed by *P. longum* fruit extracts viz., chloroform (71%), ethyl alcohol (64%) and water (65%) (Fig. 3), indicating their capability to protect liver from peroxidative damage. The other extracts accounted for lower activity (10-46%) at test concentration.

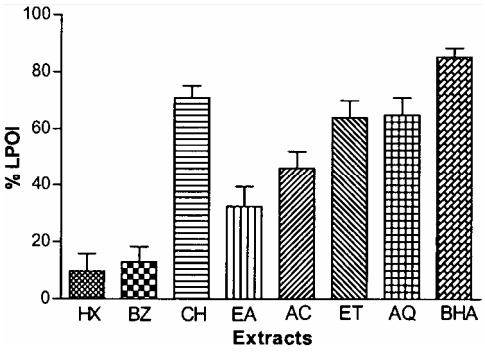

Fig. 3—Protective activity of *P. longum* fruit extracts against lipid peroxidation [Extracts were prepared in hexane (HX), benzene (BZ), chloroform (CH), ethyl acetate(EA), acetone(AC), ethyl alcohol (ET) and water (AQ) as described in 'Materials and Methods'. Lipid peroxidation inhibition (% LPOI) activity was determined in presence of 200 µg of extract in reaction mixture and BHA was used as standard. Results are expressed as mean ± SD (n = 3, $p<0.05$)]

Effect of extracts on hepatic markers in serum

The results of serum biochemical parameters in the control and various experimental groups are depicted in Figs 4-5. Administration of $AlCl_3$ in rats by oral route caused liver damage, as revealed by appreciable increase in activities of serum enzymes SGOT, SGPT and ALP (Fig. 4 A, B, C) as well as creatinine and bilirubin contents (Fig. 5), as compared with control rats. Co-administration of rats with *P. longum* fruit aqueous extract and $AlCl_3$ caused decline in altered levels of serum biochemical markers. Aqueous extract treatment showed significant protection against $AlCl_3$-induced hepatotoxicity.

Administration of Liv-52 and *P. longum* fruit aqueous extract restored the hepatic marker levels in serum. However, Liv-52 was more effective in reversing the toxic effect of $AlCl_3$ on hepatic tissue, in comparison with *P. longum* (Figs 4, 5). Administration of $AlCl_3$ significantly enhanced the serum SGOT, SGPT and ALP enzyme activities up to 219.23, 49.67 and 91.41 U/L, respectively. Treatment with fruit extract mitigated the $AlCl_3$-induced hepatotoxicity, as indicated by the decrease in serum enzymes i.e., SGOT (187.93 U/L), SGPT (44.22 U/L), and ALP (67.06 U/L) activities. *P. longum* fruit extract also decreased the $AlCl_3$-induced elevated

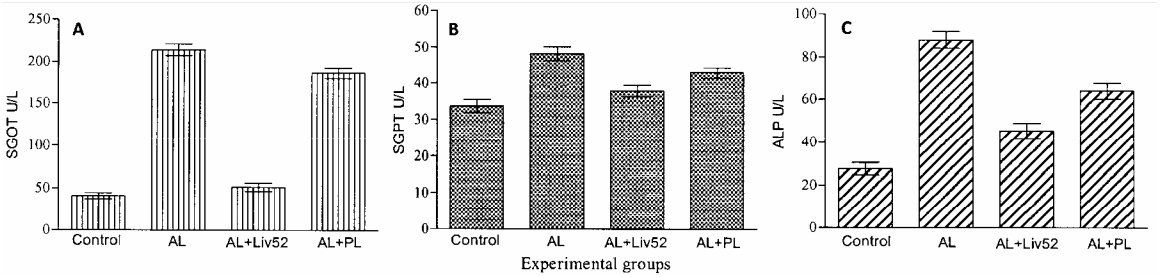

Fig. 4—Effect of *P. longum* fruit aqueous extract (PL) on (A) serum glutamic oxaloacetic transaminase (SGOT) level, (B) serum glutamate pyruvate transaminase (SGPT) level and (C) alkaline phosphatase (ALP) level in $AlCl_3$ (AL)-induced hepatotoxicity in Wistar rats [Enzyme activity was expressed as U/L. The data represent mean ± SD (n = 6; p<0.001)]

levels of bilirubin (total and direct) and creatinine in serum of experimental rats (Fig. 5).

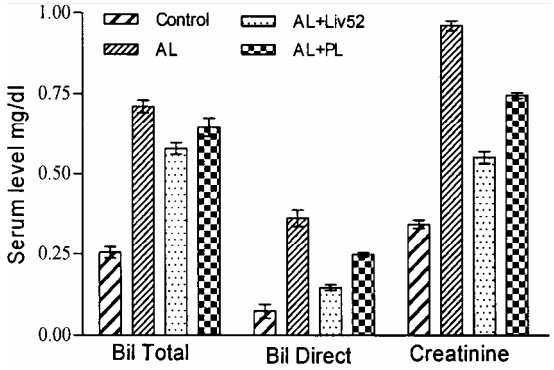

Fig. 5—Effect of *P. longum* fruit aqueous extract (PL) on serum hepatic markers in $AlCl_3$-induced hepato-toxicity [Content of hepatic markers in serum was expressed as mg/dL. *Abbreviations*: AL, $AlCl_3$; Bil, total and direct bilirubin. The data represent mean ± SD (n = 6; p<0.001)]

Discussion

Secondary metabolites of phenolic nature, including flavonoids are responsible for the variety of pharmacological activities[23]. Recently, there has been an upsurge of interest in the therapeutic potential of medicinal plants, which might be due to their phenolic compounds, particularly to flavonoids[24]. Flavonoids are a group of natural compounds with variable phenolic structures found ubiquitously in plants. The pharmacological activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure. Lipid peroxidation is a common consequence of oxidative stress. Flavanoids protect lipids against oxidative damage by various mechanisms. Because of their capacity to chelate metal ions, flavonoids also inhibit free radical generation[23].

Several flavonoids, such as catechin, apigenin, quercetin, naringenin, rutin and venoruton have shown the hepatoprotective activity[24]. Present study demonstrated significant amount of flavonoid content in most of the *P. longum* fruit fractions. The extracts also exhibited significant lipoprotective activity against iron-induced membrane damage in rat liver homogenate (Fig. 3). A positive correlation ($r^2 = 0.368$) was observed between the total flavonoid content and percentage lipid-peroxidation inhibition activity of the *P. longum* fruit extracts. Our findings were in agreement with other studies on the lipoprotective efficacy of flavonoids, showing a positive correlation between these two parameters[25,26].

Cancer chemotherapeutic agents can often provide prolongation of life, temporary relief from symptoms and occasionally complete remission. A successful anticancer drug should kill or incapacitate cancer cells by inducing apoptosis in cancer cells. Chemopreventive agents comprise diverse groups of compounds having different modes of action with ultimate ability to induce apoptosis. Understanding the mechanism of action of chemopreventive agents provides useful information for their possible applications in cancer prevention and therapy[10].

In recent years, cell cycle modulation by various synthetic and natural agents is gaining widespread attention. Disruption of cell cycle plays a key role in inhibition of cancer progression; its modulation by phytoconstituents is a directional approach in carcinogenesis prevention. A number of herbs have been reported to induce cell cycle arrest and thereby play an important role in cancer prevention and therapy[10,27].

In present study *P. longum* fruit extracts demonstrated considerable cytotoxicity against test cell lines. Some of the extracts viz., hexane, benzene and acetone exhibited significant growth inhibitory potential (up to 95%) against lung, ovary, breast, leukemia and prostate cancer cell lines (Fig. 1). The effective extracts exhibited appreciable cell cycle inhibition potential against A549 cancer cell lines. Treatment of lung cancer cell line (A549) with hexane, benzene and acetone extracts produced up to

63% increase in sub-G1 population (Fig. 2A-E), while standard anticancer drug camptothecin accounted for 48% increase in sub-G1 population. The study showed that *P. longum* extracts were potent inhibitors of A549 lung cancer cell line.

Enzymological and biochemical parameters in blood are widely used as biomarkers for assessment of the functional status of health. Alterations in liver enzyme activities (SGOT, SGPT and ALP) in the experimental animal model suggest liver dysfunction. Elevated level of the liver enzymes in blood might indicate the damage or inflammation in the liver cells[28]. The hepatic damage leads to leakage of bilirubin, creatinine and enzymes into the blood. In the present study, elevation in activity of serum enzymes ALP, SGOT and SGPT (Fig. 4 A-C), as well as bilirubin and creatinine contents (Fig. 5) was observed in $AlCl_3$-administered rats at a dose of 100 mg/kg body wt. The rise of these biomarkers in serum suggested that $AlCl_3$ induced hepatotoxicity. Co-administration of *P. longum* fruit aqueous extract with the $AlCl_3$ improved the hepatic condition, as indicated by the decrease in the level of ALP, SGOT, SGPT, creatinine and bilirubin, signifying the role of *P. longum* as hepatoprotectant. However, Liv52, a well-known hepatoprotective drug was found to be more effective in reversing the metal-induced hepatotoxic effect. The study revealed that *P. longum* fruits have anticancer and hepato-protective activities.

In conclusion *P. longum* fruit showed potent cytotoxic activity which was mediated by cell cycle arrest and *in vitro* and *in vivo* hepatoprotective activity against $AlCl_3$-induced hepatotoxicity.

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